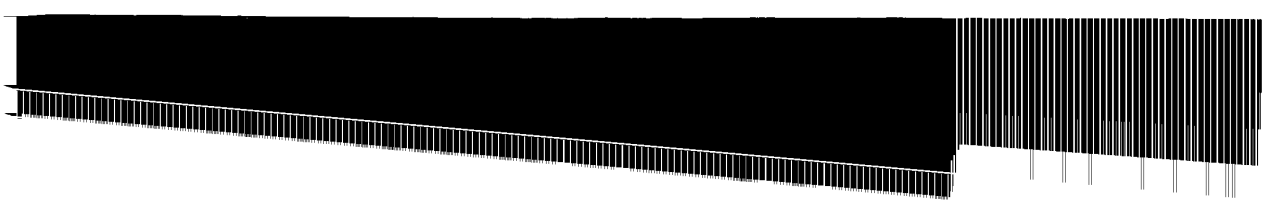

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(54) Title: ANTIHAEMOSTATIC AGENTS FROM NECATOR AMERICANUS

(57) Abstract

The invention relates to the use of excretory-secretory (ES) products of the human hookworm Necator americanus as antihaemostatic agents. In particular, the products inhibit the activity of coagulation factor Xa, and inhibit platelet aggregation.

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ANTIHAEMOSTATIC AGENTS FROM NECATOR AMERICANUS.

The present invention relates to antihaemostatic agents for the treatment of human thrombotic disorders.

Human thrombotic disorders are widely treated by the use of anticoagulant and thrombolytic drugs. For example, heparin is a common anticoagulant, whilst fibrin-rich blood clots may be dissolved using tissue plasminogen activator, streptokinase and urokinase. However, there are some side effects and other drawbacks associated with the use of the known drugs.

The present invention seeks to provide novel antihaemostatic agents.

According to the invention there is provided an excretory-secretory product of the human hookworm Necator americanus for use as an active pharmaceutical substance.

According to the invention there is provided derivatives of excretory-secretory (ES) products of the human hookworm Necator americanus for use as an active pharmaceutical substance.

The invention further provides for the use of

excretory-secretory products of the human hookworm Necator americanus for the manufacture of an antihaemostatic composition.

The invention further provides an antihaemostatic composition comprising a pharmaceutically acceptable diluent or carrier and an excretory-secretory product of the human hookworm Necator americanus.

The invention further provides an antihaemostatic composition comprising a pharmaceutically acceptable diluent or carrier and an active ingredient obtained from an excretory-secretory product of the human hookworm Necator americanus.

The active ingredient may be obtained directly, or by chemical synthesis, or by a genetic engineering technique.

The invention further provides excretory-secretory products or derivatives thereof of the human hookworm Necator americanus for use as an inhibitor of platelet aggregation.

The invention further provides excretory-secretory products or derivatives thereof of the human hookworm Necator americanus for use as an inhibitor of Factor Xa activity.

The invention further provides derivatives of excretory-secretory products of the human hookworm Necator americanus for use as an inhibitor of platelet dense granule release.

The invention will be further described for the purposes of illustration only with reference to the following examples and accompanying drawings, in which:-

Fig. 1 shows the effect of Necator americanus excretory-secretrory products on human plasma clotting times;

Fig. 2 shows the effects of Necator americanus excretory-secretory products on human plasma stypven clotting time;

Fig. 3 shows the effects of Necator americanus on human Factor Xa activity;

Fig. 4 shows the effects of Necator americanus excretory-secretory products on platelet aggregation in human plasma mediated respectively by collagen, adenosine diphosphate (ADP), thrombin and platelet activating factor (PAF);

Fig. 5 shows the effects of Necator americanus excretory-secretory products on platelet granule release; and

Fig. 6 shows the degradation of human fibrinogen by Necator americanus excretory-secretory products.

PREPARATION OF NECATOR AMERICANUS EXCRETORY-SECRETORY (ES) PRODUCTS

Necator americanus is passaged in DSN hamsters. Faecal cultured from infected animals provide infective (L3) larvae, which are then used to infect neonates percutaneously. Adult worms are routinely harvested from the small intestines of infected hamsters 5 weeks post-infection. The ileum of the infected hamster is removed, opened longitudinally, and placed in Hanks' saline at 37°C. As worms release their hold on the mucosa, they are carefully removed, thoroughly washed, and cleansed in Hanks' saline containing 100 iu/ml penicillin and 100 ug/ml streptomycin. Cleansed worms are examined under a dissecting microscope, and undamaged worms retained.

Under sterile conditions, worms are added to RPMI 1640, containing penicillin and streptomycin. The worms are then cultured for 16 hrs, and supernates removed for analysis of thrombolytic and anticoagulant activities.

Culture supernatants are filtered through 0.2 um Minisort NML filters (Sartorius) to remove eggs that may have been deposited during the culture period.

Concentration of supernatants is carried out using centrifugal separation methods. The bulk of the culture media is removed using Macrosep Centrifugal Concentrators (Flowgen) with a cut off point of 10K. Final concentration and separation into 2 fractions according to MW is carried out using Centricon micro concentrators (Amincon) with cut off points of 30K and 10K. Imidazole saline buffer (Sigma) is used to dilute the supernatant at this stage to aid in separation.

TESTS TO ASSESS ACTIVITY AGAINST THE INTRINSIC AND EXTRINSIC PATHWAYS OF BLOOD COAGULATION

The Intrinsic Pathway

This involves testing the time taken to form a fibrin polymer clot, with ample prothrombin and fibrinogen present and no activation of factor VII. The test which gives the most consistent results is the activated partial thromboplastin test.

Activated Partial Thromboplastin Test (APTT)

With the exception of Ca^{2+}, platelet rich plasma (PRP) contains all the factors necessary to activate prothrombin by the intrinsic pathway. The rate of clotting is a measure of the overall coagulant activity developed and this will be decreased if there is inhibition of any intrinsic pathway factor or factor-complex.

Both the number of platelets present in PRP and the extent of exposure to glass may vary during the test, markedly affecting the test results. To avoid these inconsistencies, platelet poor plasma (PPP) is used for the test, and to this an optimal amount of platelet substitute (phospholipid emulsion) is added. In addition, optimal glass activation is obtained by incubating with Celite.

The test is carried out in a water bath at 37°C. The 50 ul PPP in a previously unused glass test tube, 25 ul Celite (4% w/v in 0.85% NaCl) and 25 ul phospholipid substitute (Rabbit brain cephalin (RVC): 1 vial in 5 ml 0.85% NaCl) is added. (Both the Celite and RBC were obtained from Sigma Diagnostics). At intervals, during a 6 minute incubation time, the tube

is agitated to disperse the Celite. 50 ul warm d $CaCl_2$(0.025M) is then added and the timing started. At 2-3 second intervals, the tube is tilted and observed for the formation of a clot. To assess the effects of hookworm ES products on APTT, the PPP is preincubated for 10 minutes with varying concentrations of ES products (volumes being made up with saline). As delayed clotting time is often followed by the formation of a poor clot, the aggregation of Celite particles (caused by fibrin formation) is taken to indicate clot formation. If no clot is formed within 3 minutes, the timing is terminated.

Results

The ES products of N. americanus have a dose-dependent effect on APTT (see figure 1).

The Extrinsic Pathway

This involves the time taken to form a fibrin polymer clot without the presence of platelet phospholipid but with tissue factor and ample prothrombin and fibrinogen present. The test used was the Prothrombin Time Test (PT).

Prothrombin Time Test (PT)

Tissue thromboplastin (tissue factor), a phospholipoprotein, was added to human PPP to activate factor VII and to provide the tissue phospholipid needed both for factor X activation and as part of the prothrombinase complex.

The test is carried out in a water bath at 37°C.

Equal volumes of thromboplastin solution and 0.025M $CaCl_2$ are mixed and stored in the water bath. Th thromboplastin (obtained from Sigma Diagnostics - 1 vial reconstituted with 2 ml dionized water). 50 ul citrated PPP is warmed in a clean unused test tube, 100 ul thromboplastin/$CaCl_2$ mixture added, and the timing started. (The tube is tilted every 2-3 seconds and the clotting time noted).

To assess the effects of hookworm ES products on PT, the PPP is first incubated for 10 minutes with varying concentrations of ES products (volumes being made up with saline). A prolonged clotting time or no clot formation is taken as an indication of inhibition in the extrinsic pathway by hookworm ES products.

Results

The ES products of N. americanus have a dose-dependent effect on PT (see figure 1).

MEASUREMENT OF ACTIVITY AGAINST FACTOR Xa

Stypven Clotting Time Test

Stypven clotting time is the accelerated clotting time of recalcified plasma when mixed with Russell's Viper Venom (RVV). In the presence of brain phospholipid, RVV activates factor X directly.

The test is carried out in a water bath at 37°C. 50 ul human PPP is warmed in a clean unused test tube, and 25 ul RVV (in cephalin solution) added. (The RVV in cephalin was obtained from Sigma Diagnostics - 1 vial was dissolved in 3 ml 0.85% NaCl). After 3 minutes incubation, 50 ul 0.025M $CaCl_2$ is added, and the time

started.

The effect of hookworm ES products on Stypven Time is assessed by adding varying concentrations of ES products to the mixture after incubation with RVV and allowing a further incubation period of 10 minutes. 50 ul of $CaCl_2$ is then added and the timing started. Prolongation of clotting time is taken as an indication of inhibition in the common pathway of coagulation by hookworm ES products.

Results

"Necator" ES products prolong human plasma Stypven clotting time (see figure 2).

Confirmatory Fluorogenic Assay for Factor Xa activity

A synthetic oligopeptide substrate, possessing a fluorescent group commercially used to measure activated factor X, was used to confirm factor Xa activity. Factor Xa splits the AMC.HCl from the carboxyl terminal of this substrate molecule causing it to fluoresce. The enzyme activity can thus be assessed using a fluorimeter.

Boc.Ile.Glu.Gly.Arg.AMC.HCl, the substrate used for the measurement of factor Xa activity, was purchased from Novabiochem (UK) Ltd., Nottingham. This is made up to a 5 mM solution with distilled water and stored in small aliquots at -20°C. Human factor Xa was obtained from Diagnostica Astho, Deeside, Clywd.

Assays are conducated at 37°C using 50 mM Tris-HCl buffer pH 8.0 containing 100 mM NaCl and 10 mM $CaCl_2$.

Results

The release of platelet dense granules were inhibited by the preincubation of platelet products with ES products (Fig. 5)

Fibrinogenolysis

The effect of Necator ES products on fibrinogenolysis was investigated by incubating human fibrinogen and Necator ES products over timed intervals then examining the degradation products using SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), 40ug aliquots of fibrinogen were incubated with 10ug Necator ES products at 37°C for 1,3,5 and 24 hour periods. At the end of each incubation period, the samples were run under reducing conditions on a 7-12% gradient gel. This was run at 30volts overnight, at 200volts until completion, and then stained with Coomassie brilliant blue R-250.

Results

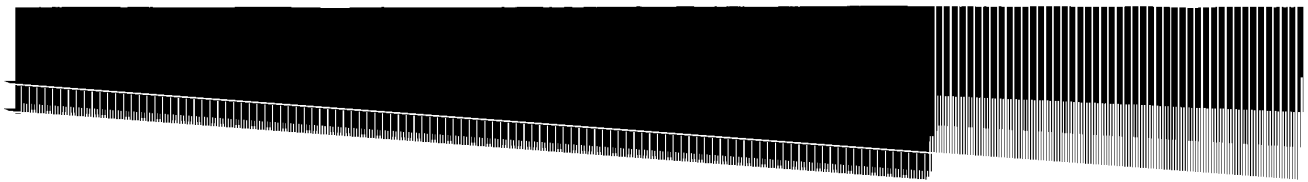
Fibrinogenolysis occured during incubation of human fibrinogen with Necator americanus ES products (Fig. 6). After one hour incubation, degradation of fibrinogen was clearly seen. Prolonged incubation times

caused increading amounts of degradation, and after 24 hours most of the 40ug protein sample was degraded.

It is thus shown that excretory-secretory products of the human hookworm prevent haemostasis by various strategies, including inhibition of coagulation factor Xa, a pivotal component of the clotting cascade. Platelet activation is also affected.

Products derived from a human parasite would not be expected to give rise to compatability problems when administered to humans.

Whilst endeavouring in the foregoing specification to draw attention to those features of the invention believed to be of particular importance it should be understood that the Applicant claims protection in respect of any patentable feature or combination of features hereinbefore referred to and/or shown in the drawings whether or not particular emphasis has been placed thereon.



Claims:-

1. An excretory-secretory product of the human hookworm Necator americanus for use as an active pharmaceutical substance.

2. Derivatives of excretory-secretory (ES) products of the human hookworm Necator americanus for use as an active pharmaceutical substance.

3. The use of excretory-secretory products of the human hookworm Necator americanus for the manufacture of an antihaemostatic composition.

4. An antihaemostatic composition comprising a pharmaceutically acceptable diluent or carrier and an excretory-secretory product of the human hookworm Necator americanus.

5. An antihaemostatic composition comprising a pharmaceutically acceptable diluent or carrier and an active ingredient obtained from an excretory-secretory product of the human hookworm Necator americanus.

6. A composition according to Claim 5, wherein the active ingredient is obtained either directly or by

chemical synthesis or by a genetic engineering technique.

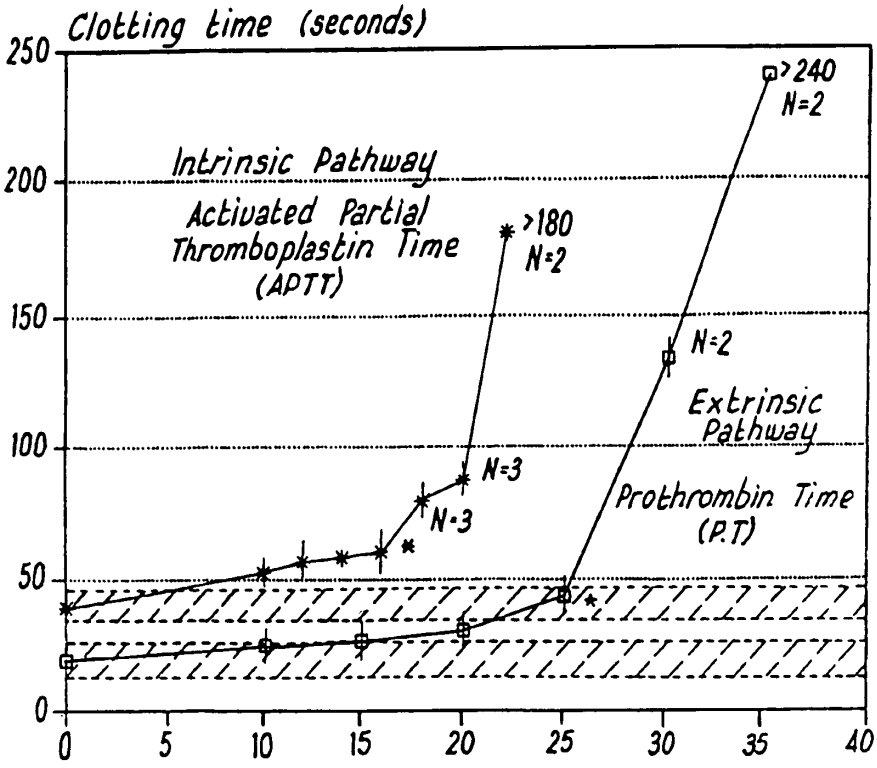
7. Excretory-secretory products or derivatives thereof of the human hookworm Necator americanus for use as an inhibitor of platelet aggregation.

8. Excretory-secretory products or derivatives thereof of the human hookworm Necator americanus for use as an inhibitor of Factor Xa activity.

9. Excretory-secretory products of the human hookworm Necator americanus for use as an inhibitor of platelet dense granule release.

10. Any novel subject matter or combination including novel subject matter disclosed, whether or not within the scope of or relating to the same invention as any of the preceding Claims.

Effect of Necator americanus ES Products on Human Plasma Clotting Times



Shaded area denotes normal activity range

Clotting Test

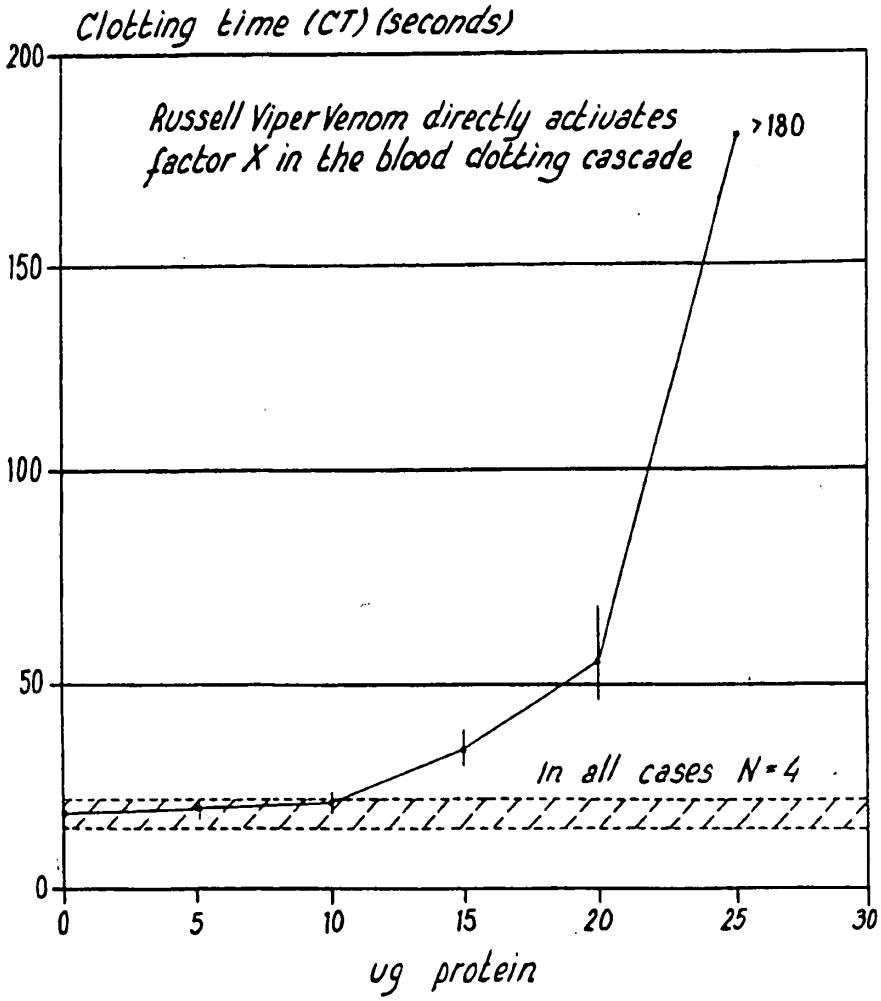
—✱— APTT —□— PT

Unless otherwise stated, APTT: N=4 PT: N=5

Preincubation at 37 deg.C with Necator ES prolongs APTT & PT · Denotes conc. when clots start to become very small.

FIG. 1

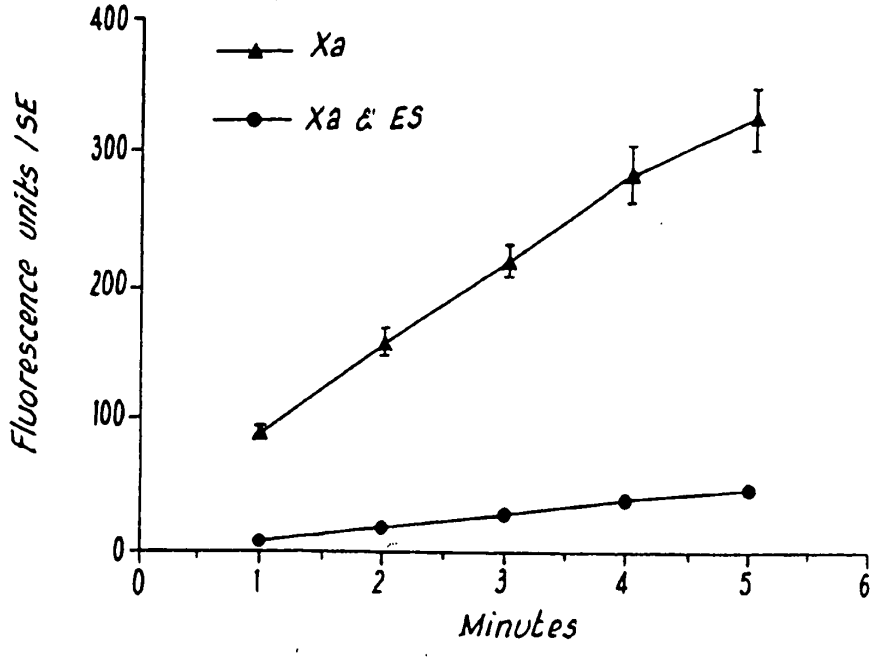
Effects of Necator americanus ES Products on Human Plasma Stypven Clotting time.



Incubation at 37 deg.C of factor X8 with N. americanus ES products prolongs clotting time.

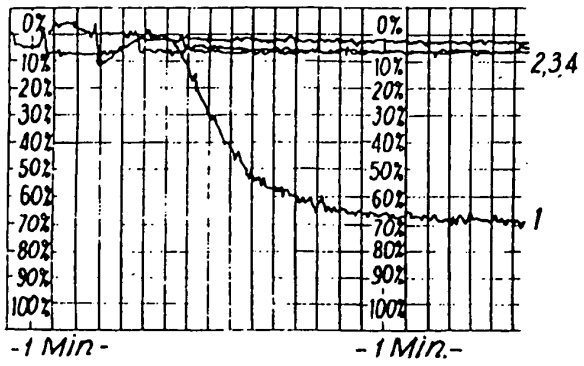
Shaded area denotes normal activity range

FIG. 2

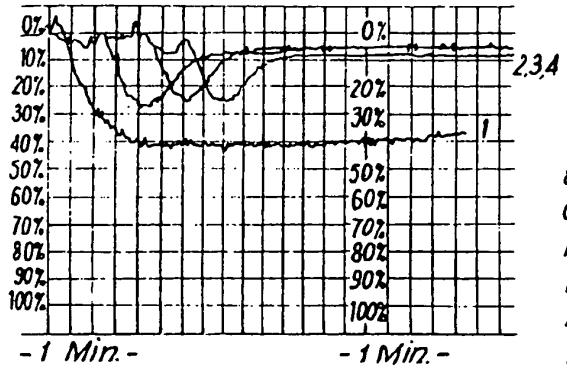


Confirmation of the effects of Necator americanus on human factor Xa.

FIG. 3



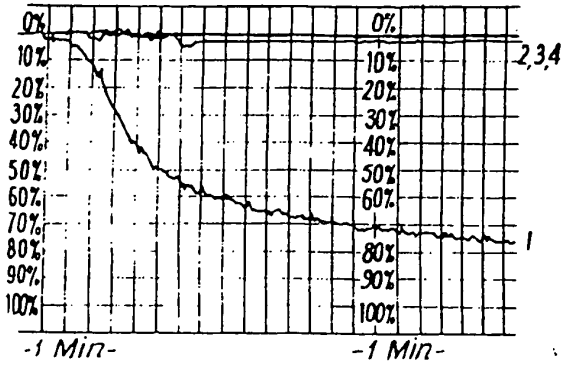
(a) Collagen



(b) ADP

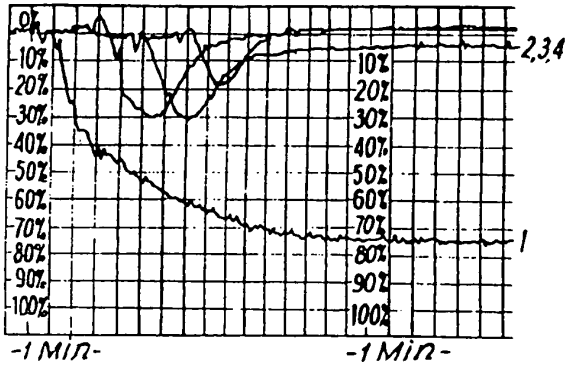
Effects of Necator americanus ES products on Platelet Aggregation in Human Plasma Mediated by Collagen. Adenosine Diphosphate(ADP), Thrombin, and Platelet Activating Factor (PAF).

(C)



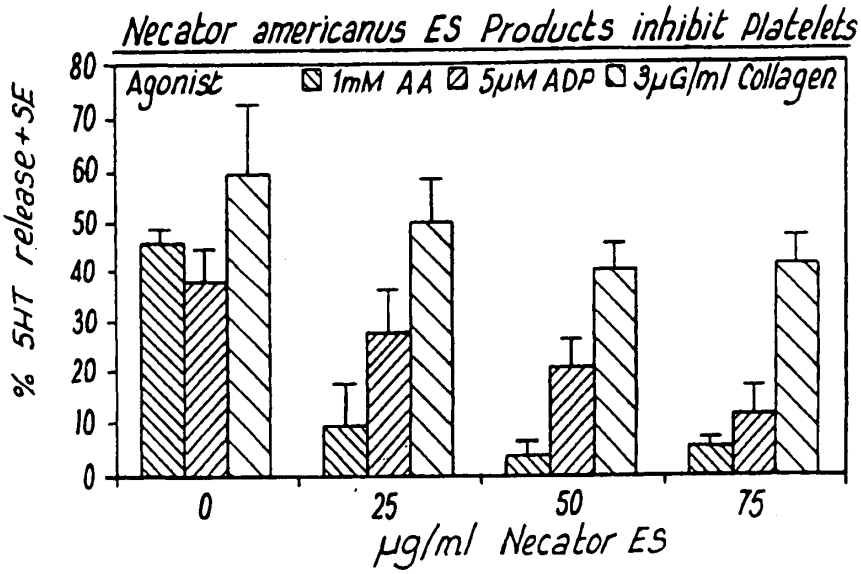
Thrombin (washed platelet preparation)

(d)



PAF

Fig. 4



-The effects of Necator americanus ES products on platelet dense granule release.-

FIG.5

Hookworm secretory_proteases

N. americanus ES products degrade human fibrinogen in a time dependent manner.

Fibrinogen alone was used as a control.
Lane 1: Markers
Lane 2: Fibrinogen
Lane 3: 1 hr. incub.
MW: 68.000 (Ax)
52.000 (Bβ)
47.000 (γ)
Lane 4: 3hr. incub.
Lane 5: 5hr. incub.
Lane 6: 24hr. incub.

Fig. 6

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/02406

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/435 A61K35/56

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INTERNATIONAL JOURNAL FOR PARASITOLOGY, vol.22, no.5, 1992, OXFORD,GB pages 563 - 572 KUMAR ET AL 'THE PARTIAL CHARACTERIZATION OF PROTEASES PRESENT IN THE EXCRETORY/SECRETORY PRODUCTS AND EXSHEATHING FLUID OF THE INFECTIVE (L3) LARVA OF NECATOR AMERICANUS'	1,2,4-9
A	see the whole document ---	3
X	PARASITE IMMUNOLOGY, vol.13, no.2, March 1991, OXFORD,GB pages 187 - 199 PRITCHARD ET AL 'NECATOR AMERICANUS SECRETORY ACETYLCHOLINESTERASE AND ITS PURIFICATION FROM EXCRETORY-SECRETORY PRODUCTS BY AFFINITY CHROMATOGRAPHY'	1,2,4-9
A	see the whole document --- -/--	3

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 March 1995	27 -03- 1995

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Sitch, W

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 94/02406

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PARASITOLOGY, vol.100, no.PT.2, April 1990, CAMBRIDGE,GB pages 317 - 326 PRITCHARD ET AL 'EPIDEMIOLOGY AND IMMUNOLOGY OF NECATOR AMERICANUS INFECTION IN A COMMUNITY IN PAPUA NEW GUINEA:HUMORAL RESPONSES TO EXCRETORY-SECRETORY AND CUTICULAR COLLAGEN ANTIGENS'	1,2,4-9
A	see the whole document ---	3
X	PARASITE IMMUNOLOGY, vol.9, no.2, March 1987, OXFORD,GB pages 219 - 234 CARR ET AL 'ANTIGEN EXPRESSION DURING DEVELOPMENT OF THE HUMAN HOOKWORM,NECATOR AMERICANUS (NEMATODA)'	1,2,4-9
A	see the whole document ---	3
X	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol.19, no.3, 1986, AMSTERDAM pages 251 - 258 CARR ET AL 'IDENTIFICATION OF HOOKWORM (NECATOR AMERICANUS) ANTIGENS AND THEIR TRANSLATION IN VITRO'	1,2,4-9
A	see the whole document ---	3
X	BEHNKE ET AL 'HUMAN PARASITIC DISEASES.VOLUME 4.HOOKWORM INFECTIONS (GILLES AND BALL,EDS.).9.AN OVERVIEW.PAGES 217-237' 1991 , ELSEVIER SCIENCE PUBLISHERS , AMSTERDAM	1,2,4-9
A	see page 221 -----	3

INTERNATIONAL SEARCH REPORT

.ational application No.
PCT/GB94/02406

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
The scope of claim 10 is not ascertainableto the extent that a meaningful search can be carried out thereon.
See Article 6 PCT.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.